HEAT PRODUCTION BY THE EGGS OF *ARBACIA PUNCTULATA* DURING FERTILIZATION AND EARLY CLEAVAGE.

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The general problem of development has in it so much of the unexplained that any attempt to add to our information by attacks from new directions may seem warranted. The work here reported was first undertaken several years ago, but was not carried to a successful issue until this past summer because of experimental difficulties unforeseen at the start.

The work was originally undertaken in an attempt to check the work of Warburg and of Loeb and Wastenys concerning the oxygen consumption of eggs before and after fertilization. It will be recalled that these investigators had found that immediately after fertilization there occurs a remarkable increase in the rate of oxygen consumption, amounting to 4 to 6 times the amount used before fertilization. It is to be assumed that there is also a corresponding increase in the carbon dioxide production of the eggs. This latter would, of course, be exceedingly difficult to measure in the case of marine eggs. If there is any considerable increase in oxygen consumption following fertilization there should be a corresponding increase in the amount of heat produced by the eggs as a result of the oxidation process. The question to be faced was whether, with the facilities at our command, we would be able to make a series of measurements which would bear examination by a physicist. Preliminary tests made in 1920 and 1921 indicated that the production of heat by fertilized eggs was a measurable quantity. These tests, observed in the latter year by a group of physicists who were visiting the Marine Laboratory, proved to be sufficiently encouraging to warrant further expenditures of time and money.

[1] Contribution from the Zoölogical Laboratory of Oberlin College, and from the Marine Biological Laboratory, Woods Hole, Mass. To the Director and other officials of the Marine Biological Laboratory we express our thanks for many courtesies extended to us.

Since this investigation was first projected others have made studies of a somewhat similar character. Myerhof (6) measured the heat production of segmenting Echinoderm eggs by means of a Beckmann thermometer, using a large vacuum flask for his calorimeter. Certain irregularities that occur in his curves may be due to experimental error, but suggest changes in the rate of heat production at different stages of development. Since then Shearer (7) has carried out similar measurements in connection with his work on the oxidation processes of Echinoderm eggs and finds that the rate of heat production after fertilization is constant for at least ten or twelve hours. He makes the statement that readings were taken at fairly frequent intervals at the commencement of the experiment, and at intervals of several hours after that. In view of this statement it seemed advisable to us to repeat the work making frequent readings and using methods of higher precision.

Possible Sources of Error.

In an investigation involving the measurement of such slight temperature changes as are expected here care must be taken to foresee and provide against all possible heat transfers into or out from the experimental flasks or to know the magnitude of such transfers. In any event such heat losses or gains should be small as compared with the total heat production which it is desired to measure. In our experiments the following possible channels of heat transfer existed, and were checked: Conduction to or from the water in the flasks

(1) by the air in the mouth of the flask,
(2) by means of the glass forming the neck of the flask,
(3) along the main thermopile,
(4) along the secondary thermopile,
(5) along the tubes of the stirring apparatus,
(6) along the fertilizing tube,
(7) by changes in the temperature of the water surrounding the flasks.

There are also possibilities of error arising from lack of care in controlling the conditions environing the electrical apparatus. Among these may be mentioned:

1. Mechanical jarring of the galvanometer used in making the measurements.
2. The temperature of the room must be maintained as nearly constant as possible during the course of an experiment. It was found to be especially necessary to avoid the possibility of drafts of air striking the apparatus.
3. It was found necessary to shield the electrical apparatus from stray electrical currents. This was found to be of the greatest importance following a severe electrical storm.
4. It was found wise to avoid all stress and strain in the wires of the thermopiles, such as might be caused by too much bending of the wires, or placing tension upon them.

It was found possible to obviate much of the possibility of the errors of the first group by using a water cap, designed so as to provide a current of water coursing continuously over the experimental flask as well as around it.

Apparatus and Methods of Work.

The general method employed was that of the micro-calorimeter, developed by Hill in his work upon muscle. Fig. 1 shows the experimental set-up used. This method has one serious disadvantage. Since corrections for heat loss are dependent upon the temperature difference and the time, long runs can not be made, since these corrections soon become a very large part of the result. In this work the corrections could be kept less than 15 per cent. of the total temperature change during a period of two and a half or three hours. Two straight sided commercial vacuum flasks of about 75 cc. capacity were used, (I.) containing 50 cc. of the egg suspension, and (II.) an equal quantity of water. These flasks had been especially exhausted through the kindness of Dr. W. R. Whitney of the Research Laboratory of the General Electric Company. When flask (I.) contained 50 cc. of water it had a heat loss of 16 calories per hour per degree difference in temperature between the interior and the exterior. The flasks used by Hill (2) had a loss of about 12 calories per hour for 250 cc. and those used by Shearer (7) about 19 calories per hour for 800 cc. This small loss for a flask of such small capacity is evidence of the great value

of very careful evacuation. The flasks were submerged in running sea water to within a short distance of their tops, and a water cap (*d*) made according to a design by White (9) was

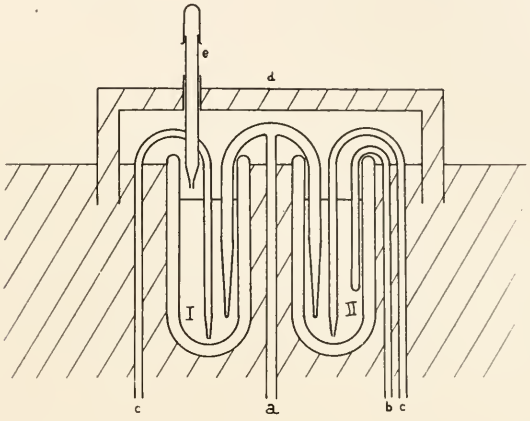

FIG. 1. Diagram of experimental set-up. I. and II., Vacuum flasks. *a*, main thermopile. *b*, auxiliary thermopile. *c*, *c*, stirring tubes. *d*, water cap. *e*, sperm pipette.

placed over them. A pipette (*e*) projected through the cap and was arranged so that "dry sperm" could be held in it, and then mixed with the contents of flask (I.) whenever desired. A continuous flow of water was maintained through this cap so that the whole formed a uniform temperature enclosure. The sea water temperature remained fairly constant except for periods of about four hours after sunrise and sunset. The variation of temperature never exceeded 0.001° C. during the period occupied by an experiment.

STIRRING APPARATUS.

It has been noted by many investigators that eggs of Echinoderms must not be heaped upon each other if normal development is to take place. In our work it was desirable to use as large a number of eggs as possible in order to get the largest possible temperature change. It was therefore necessary to devise some method of stirring the eggs which would render it impossible for them to settle to the bottom of the flask and remain there for

any considerable length of time. By the more constant stirring the eggs would at all times be able to get their needed supply of oxygen and to get rid of carbon dioxide. Work previously done by one of us (R.) had shown that stirring the water in the experimental flasks once every two or three minutes by means of an ordinary pipette was sufficient to allow the eggs to go through a normal cleavage. Such stirring was not sufficient to ensure that the water in the flask would be of uniform temperature throughout. It seemed necessary, therefore, to devise some automatic stirring device which would keep the water thoroughly stirred at all times during the course of an experiment. As a matter of interest it was found during the course of the experimentation that a failure of the stirring apparatus for as much as three minutes could be detected by a marked variation in the galvanometer readings.

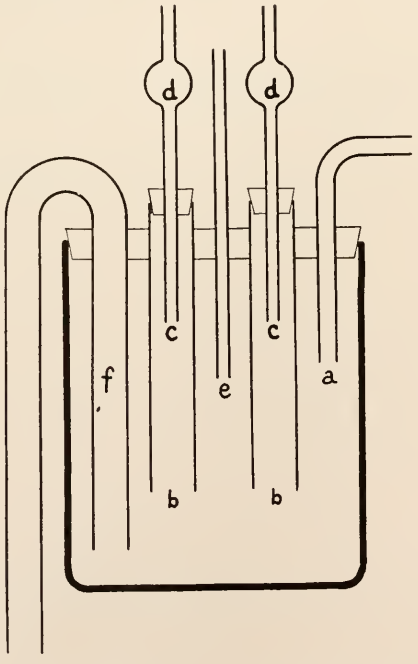

FIG. 2. Stirring apparatus. Details described in text.

The stirring was accomplished as follows: Saturated air was bubbled from the jets (*c*, *c*) at the bottoms of the flasks, by

means of the automatic intermittent siphon device shown in Fig. 2. A steady stream of water flowed in at (*a*) and as the water level rose in the bottle, air was trapped in (*b*, *b*) and forced out through (*d*, *d*). When the water reached the lower end of (*e*) it rose in the tubes, but not in the bottle (since the stopper was air tight) until (*f*) was filled beyond the bend. Then (*f*) acted as a siphon and emptied the bottle, allowing (*b*, *b*) to drain, and finally draining itself. Then the process started over again. Short pieces of rubber tubing cut at an angle were slipped over both ends of (*f*) and the lower ends of (*b*, *b*) to help in breaking the meniscus and draining these tubes. The quantity of air sent over each time could be varied by adjusting the height of the small tubes (*c*, *c*) in (*b*, *b*), and the period of the apparatus could be changed by raising or lowering (*e*). The small bulbs (*d*, *d*) prevented drops of water from being forced over into the wash bottles. These wash bottles had inlet tubes of small cross section, and were submerged in the same bath with the flasks. They served to complete the saturation of the air, if it were not already saturated, to bring it to the temperature of the bath before it went into the flasks through (*c*, *c*), and to act as a trap, preventing the suction of liquid out of the flasks through (*c*, *c*) when the stirrer bottle was emptying. In this work 7 cc. of air was sent through each flask every fourteen seconds. During a six-day continuous run, this apparatus did not vary from its average period by more than 2/5 second, always delivering the same and equal quantities of air to the flasks.

Electrical Equipment.

The electrical equipment used in these experiments consisted of the main and secondary thermopiles, galvanometer, potentiometer, storage battery cells, Weston Standard Cell, switches, etc. The main thermopile (*a*, Fig. 1) was composed of twenty pairs of copper-constantan junctions, and gave 733 microvolts per degree Centigrade, by direct calibration. (The constantan wire used was that manufactured by the Driver-Harris Wire Company, and sold under the trade name "Advance.") The reason for the unexpectedly low value of the thermopile was not found. The junctions were enclosed in thin glass tubes

filled with naphthalene and the lag of the couple was less than twelve seconds. The auxiliary or secondary thermopile (*b*) had five pairs of junctions and gave the temperature difference between flask II. and the surrounding bath. The e.m.f. developed by the couples was measured by a "White" potentiometer. This instrument, manufactured by Leeds and Northrup, gives dial readings by single microvolts and is so arranged that the resistance in the galvanometer circuit remains constant. This fact makes it possible to read fractions of a microvolt with the galvanometer. In this work one microvolt gave a deflection of 18.5 mm. on the galvanometer scale, so there were about 13,550 mm. per degree. With the high magnification telescope used it was easily possible to estimate fifths of a millimeter on the galvanometer scale. The galvanometer was of the D'Arsonval type, also manufactured by Leeds and Northrup. Its resistance was 13 ohms, period 5 seconds, and sensitivity 10^{-8} amperes per cm. with the scale a little over three meters distant. The total resistance of the galvanometer circuit was 53 ohms, which was an ohm more than the critical damping resistance. In spite of this fact there were never any oscillations, even when the sperm were introduced into the experimental flask,—only what seemed to be a very steady and somewhat rapid rise. The galvanometer circuit was shielded as far as possible, as suggested by White (10) to prevent the entrance of leakage currents into the circuit. It was, however, impossible to shield the thermopiles effectively since their leads were carried through the running sea water and very strange and erratic e.m.f.s were introduced into the circuit if the shielding system for the potentiometer and galvanometer had anything to do with the salt water or any of the piping in the room. Care had to be taken not to use for connections any wire that had been unduly bent or in any other way maltreated so as to destroy its homogeneity. On the whole the electrical apparatus worked very satisfactorily.

It was our original plan to use Hill's ingenious application of vacuum flasks to the twin calorimetric method (2) but it was found that for our small flasks the heat loss did not remain even approximately constant as the volume of the contents was varied. It was also impossible to obtain accurate values for the

conduction coefficients from cooling curves for large intervals, since Newton's law of cooling applies to small and constant temperature differences, *i.e.*, equilibrium conditions. This requirement could not be met since there was not available any current measuring device of sufficient accuracy to allow the use of electrical heating. It was necessary therefore, to get the cooling corrections under the actual experimental conditions. Following White (9)

Let θ_1 = temperature of flask (1),
θ_2 = temperature of flask (2),
θ_3 = temperature of external bath.

The temperature coefficient of conduction for the flask is defined as the temperature change between the inside and the outside of the flask in unit time when the temperature difference between the inside and the outside is unity. This may be written in mathematical form for flask (I.), $K_1 =$; and for flask (II.), $K_2 =$.

$$K_1 = \frac{1}{\theta_1 - \theta_3} \cdot \frac{d\theta_1}{dt},$$

$$K_2 = \frac{1}{\theta_2 - \theta_3} \cdot \frac{d\theta_1}{dt}.$$

Similarly, for the heat conduction between the flasks along the thermopile,

$$k_1 = \frac{1}{\theta_1 - \theta_2} \cdot \frac{d\theta_2}{dt},$$

$$k_2 = \frac{1}{\theta_2 - \theta_1} \cdot \frac{d\theta_2}{dt}.$$

Also let w_1 and w_2 equal the temperature changes due to stirring and evaporation. Then considering temperature changes where there is no liberation of heat in either flask

$$\frac{d(\theta_1 - \theta_2)}{dt} = K_1(\theta_1 - \theta_3) - K_2(\theta_2 - \theta_3) + k_1(\theta_1 - \theta_2) - k_2(\theta_2 - \theta_1) + w_1 - w_2 \quad (1)$$

$$= (K_1 + k_1 + k_2)(\theta_1 - \theta_2) + (K_1 - K_2)(\theta_2 - \theta_3) + w_1 - w_2.$$

$\theta_1 - \theta_2$ is proportional to θ_a, the e.m.f. of the main thermopile,
$\theta_2 - \theta_3$ is proportional to θ_b, the e.m.f. of the auxiliary thermopile.

So we may write

$$\frac{d\theta_a}{dt} = K_a\theta_a + K_b\theta_b, \tag{2}$$

since both theoretically and experimentally, $w_1 - w_2 = 0$ in (1). If the quantity of heat H is liberated in (1) and the heat capacity (water plus the water equivalent of the flask) is c, then

$$\frac{d\theta_a}{dt} = \frac{1}{c}\frac{dH}{dt} - K_a\theta_a - K_b\theta_b$$

and

$$\begin{aligned} H &= c\left\{\int_{\theta_0}^{\theta_a} d\theta + K_a\int_{\theta}^{t}\theta_a dt + K_b\int_{\theta}^{t}\theta_b dt\right\} \\ &= c(\theta_a - \theta_b) + c\left\{K_a\int_{0}^{t}\theta_a dt + K_b\int_{0}^{t}\theta_b dt\right\}, \end{aligned} \tag{3}$$

where θ_0 is the value of θ_a when $t = 0$.

Independent runs were made to determine the values of K_a and K_b. With θ_b small as compared to θ_a, $d\theta_a/dt$ was determined over the range for the values of θ_a used and found to be linear in θ_a. The same was done for θ_b with θ_a small. With 50 cc. in both flasks the values obtained were

K_a = .0046 microvolt per minute per microvolt difference,
K_b = .0014 microvolt per minute per microvolt difference.

The water equivalent of the flasks was found to be 8 cc. so that $c = 58$ cc.

A Typical Experiment.

The preparations for an experimental determination of the heat production of the eggs of *Arbacia* involved a variety of considerations not usual in ordinary experimental work in zoölogy. By careful tests the running sea water of the laboratory had been found to be the most satisfactory form of available thermostat, the temperature of the water changing only very slightly during any experimental period—usually only in thousandths of a degree. Care was, therefore, taken to have all glassware and implements used at the temperature of the sea water. Flasks, pipettes, graduates, beakers, finger-bowls, dissecting instruments, wash bottles of the stirring apparatus, thermopiles, water-cap and the animals to be used were all left in running sea water of uniform temperature for some time before

the beginning of the experiment. Whenever possible all of the eggs used in an experiment were taken from a single female. These were allowed to stand for a few minutes in a finger bowl in a little more than 50 cc. of sea water. The finger bowl was floated in running sea water. Remnants of the ovary and other debris were removed from the finger bowl by forceps or pipette. After a few minutes the eggs were stirred in the water so as to be evenly distributed throughout the whole mass of water and exactly 50 cc. of the suspension was transferred to flask (I.) by means of a volumetric pipette. Also 50 cc. of sea water were placed in flask (II.). The temperature of flask (II.) was then made enough higher than that of flask (I.) so that at the end of the run it would be about as much below (I.) as it was above at the beginning. This made the value of the integral involving θ_a approximately zero and kept the maximum value of the corrections low. The water-cap with a drop of dry sperm in the fertilizing tube was then put into place, the cap filled with water and the readings begun. The starting of the experiment and the making of the observations and recording them occupied the full time of two persons. Readings of the main thermopile, θ_a, were made every 60 seconds, and of the auxiliary thermopile, θ_b, every five minutes, for a period of from two and a half to three hours. Experiments were in only a few cases continued beyond the three hour limit. When steady conditions had been reached and enough readings had been taken so that the heat production of the unfertilized eggs could be determined, the pipette was lowered until its tip was below the surface of the egg suspension, the sperm washed out and stirred into the suspension, and the pipette raised again. This operation seldom caused an irregularity of more than 0.0005° C. At the same time a sample of the same batch of eggs was fertilized in a finger bowl, and kept surrounded by running sea water. These eggs were examined from time to time. The galvanometer zero and the storage cells for the potentiometer were checked frequently. At the end of the run the average diameter of the eggs was measured, in addition to the usual data on the percentage of fertilization and development. The volume of the single egg computed from the average value of the diameter, and the total volume of eggs

used was determined by centrifuging the suspension. After the centrifuging, the eggs were deformed so that their volume represented almost all of the volume measured; thus it was possible

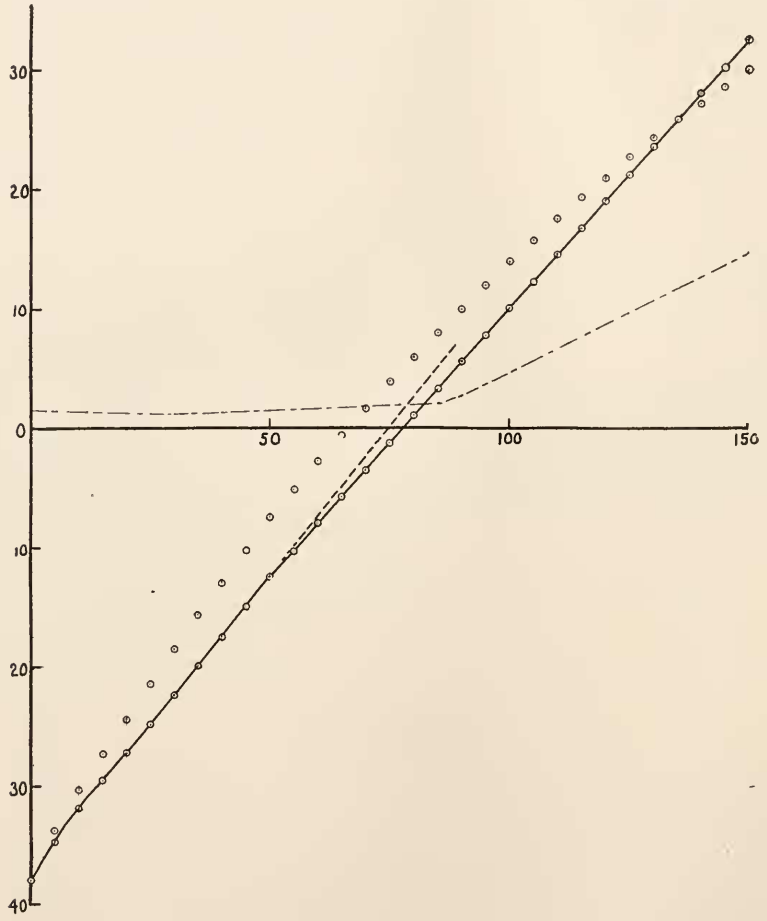

FIG. 3. Typical experimental curve. Heat production of *Arbacia* eggs. Abscissæ, time in minutes after fertilization. Ordinates, temperature differences in micro-volts. $1\mu v = 0.001364°$ C. ⊙ θ_a observed values. — – — θ_b observed values. —⊙— corrected curve. — — — projection of corrected curve of 20 to 50 minute period beyond the 50 minute position.

to obtain an estimate of the total number of eggs used. This method of counting was checked against a dilution method, similar to that employed for counting blood corpuscles, and it

was found that the agreement was very close, and that the eggs showed less variation than did the number of eggs in the fractions counted.

The course of a typical run is shown in Fig. 3. In it the average diameter of the eggs was 74 microns, and the total

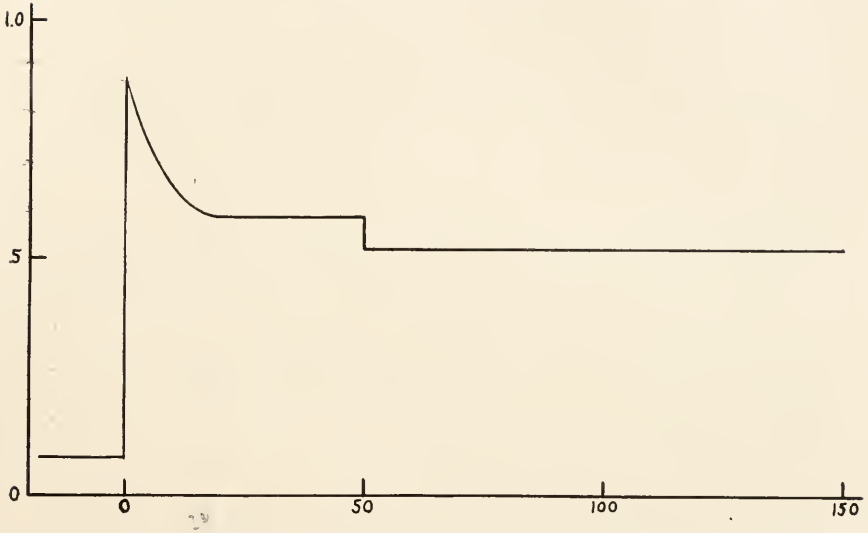

FIG. 4. Rate of heat production, *Arbacia* eggs. Abscissæ, time in minutes after fertilization. Ordinates, rate of heat production in calories per hour per million eggs.

volume was 0.82 cc. The total number of eggs was, therefore, about 3.9 millions. About 96 per cent. of these eggs were fertilized and of those fertilized about 85 per cent. were in the eight cell stage, and the rest in a late four cell stage and going into the eight cell stage so rapidly that an accurate percentage could not be obtained, when the experiment was concluded. In Fig. 3 every fifth reading is plotted. The corrected curve was obtained by taking approximate values of the integrals of equation (3) for five minutes intervals. The greatest variation in the different runs is in the behavior during the first twenty minutes after fertilization. This is probably due to the fact that the amount of sperm could not be made proportional to the number of eggs, and that the heat production of the sperm is not negligible during this period, as will be shown later. In this

respect the run shown in Fig. 3 shows a marked variation from the average.

The average of seven runs is shown in Fig. 4, which gives the approximate *rate* of heat production. The rate of heat production per million unfertilized eggs is about 0.08 calories per hour, while for the fertilized eggs after they have gone into the two cells stage it is about 0.52 calories per hour.[1] Both of these values are higher than those obtained by Shearer and by Myerhof with other sea urchins, but the ratio of fertilized to unfertilized eggs is the same. It should be pointed out that the results here given must be taken as indicative rather than conclusive and that further painstaking work is necessary.

One feature of the curve shown in Fig. 4 deserves special comment. It will be noted that the greatest rise in temperature, *i.e.*, the greatest period of heat production occurs immediately upon fertilization. This certainly raises again the question as to whether the process of membrane elevation depends upon an oxidative process, set up by the sperm cell when it comes into contact with the surface of the egg.

It must be mentioned here that Loeb (3) had expressed in 1906 the view that the essential feature (or possibly one of the essential features) of the process of fertilization is the increase in the rate of oxidation in the egg, and that this increase is caused by the membrane formation. Both Warburg and Loeb and Wasteneys had shown that the rate of oxidation in the sea-urchin egg is increased from 400 per cent. to 600 per cent. upon the entrance of the spermatozoön—and that membrane formation alone, induced by artificial means, has the same result. There is, therefore, a definite relation existing between membrane formation and increased rate of oxidation. From Warburg's (8) work it also seems likely that the increased oxidation occurs chiefly at the surface of the egg. The fact that the greatest heat production by the egg comes immediately after fertilization seems to us to make it plausible to say that the entrance of the spermatozoön induces a cortical oxidation process, and that this process results in the elevation of the fertilization membrane. The almost explosive character of the heat evolution seems to

[1] This involves an energy liberation of approximately 1 erg per egg per hour.

indicate that the oxidative process is of fundamental importance in the series of fertilization phenomena.

In the only run made upon sperm, ten drops of dry sperm were placed in the pipette and mixed with 50 cc. of sea water after steady conditions had been established. The heat produced can be expressed quite accurately by an equation of the form

$$H = H_0(1 - e^{-bt}),$$

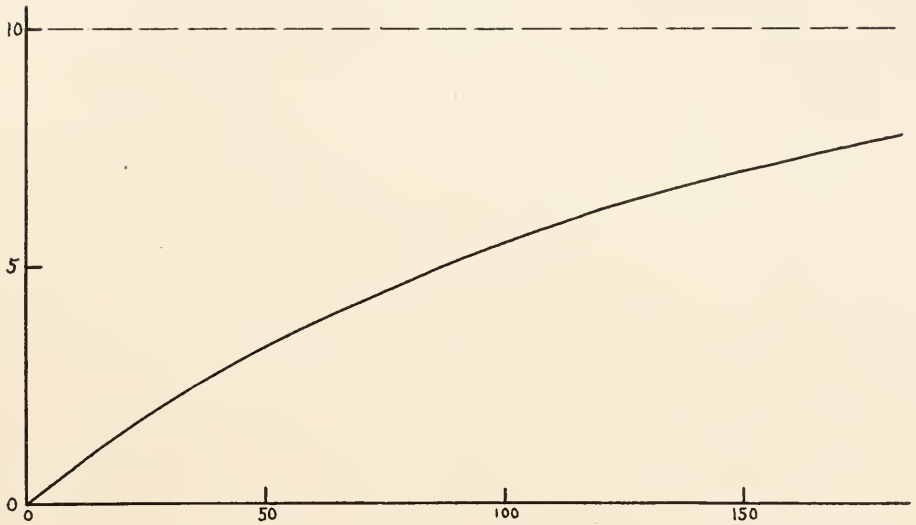

FIG. 5. Heat production of *Arbacia* sperm. Abscissæ, time in minutes after one drop of sperm is placed in 50 cc. sea water. Ordinates, temperature difference in micro-volts.

where H_0 is the total amount of heat produced—which, from the work of Cohn (1) is probably a constant for any given amount of sperm, b is probably dependent upon the pH of the water and the temperature, e is the natural base of logarithms, and t is the time after the sperm comes in contact with the water. This equation suggests very much the heat production by an exothermic reaction of the first order. In this case for ten drops of sperm,

$$H_0 = 0.79 \text{ calories},$$
$$b = 0.008 \text{ when } t \text{ is in minutes}.$$

Figure 5 shows the temperature change when one drop of sperm (approximately the amount used) is added to 50 cc. of

sea water. The curve approaches 10 micro-volts as an asymptote, but reaches 50 per cent. of that value in about 90 minutes. From this it will be seen that if there is an excess of sperm it may seriously affect results during the first twenty or thirty minutes.

As has been pointed out this method can not be used over long periods of time with the desired degree of accuracy. It also has the disadvantage of requiring large numbers of eggs, so that the longer they run, the more they tend to fall out of step, and so tend to mask any effect that may be present. This latter difficulty will remain, of course until it is possible to work with a single egg. The apparatus is being redesigned so that it will be possible to follow the heat production of both eggs and sperm under varying conditions over longer periods of time. It is planned to extend the work so as to include other forms.

Summary.

The heat production of the eggs of *Arbacia punctulata* has been measured before, during, and following fertilization, through development into the eight cell stage. It has been found that the rate of heat production at the instant of fertilization is ten to twelve times that of the unfertilized egg. After fertilization the rate of heat production decreases constantly for twenty minutes, when it reaches about 65 per cent. of the value at fertilization, and remains constant until the first cleavage, at about 50 minutes after fertilization. At the first cleavage the rate drops suddenly by more than 10 per cent., and then remains constant until the eggs are in the eight cell stage, which is as far as the work has been carried. The rate of heat production of the unfertilized eggs was found to be about 0.08 calories per hour per million eggs, and that of the fertilized eggs about 0.52 calories per hour per million eggs after the one cell stage.

An experiment on *Arbacia* sperm indicates that when placed in contact with sea water, its heat production is similar to that of an exothermic chemical reaction of the first order.

The suggestion is offered that the heat evolution occurring immediately upon fertilization is the result of an oxidative process which takes place in the cortex (chiefly) of the egg and which leads to the elevation of the fertilization membrane.

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